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13. ABSTRACT (Maximum 200 Words)

The objective of this project is to characterize the anti-tumor immunologic response to cryosurgery, a new minimally invasive approach to the ablation of breast cancer. The work consists of both murine and human studies, expanding upon resports that cryosurgery of primary tumors has been reported to be capable of developing specific anti-tumor immunological responses that can prevent the growth of micrometastasis. Because of conflicts with other grant support, the grant was relinquished effective August 31, 2003. While the project is ongoing, several interesting findings were discovered. Murine studies, utilizing the MT-901 mammary adenocarcinoma cell line in BALB/c mice, demonstrated a Th1 cytokine response to cryoablation as compared to surgical excision. Mice treated with cryoablation had long-term tumor specific memory as demonstrated by tumor re-challenge. Immunologic studies demonstrated an early but brief antibody response, a regional T-cell response and a systemic NK cell response, although no long-lasting systemic T-cell response could be identified. A manuscript is presently in preparation. Human immunologic studies have been initiated, but the results are too early for interpretation at this time.

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Introduction

With the demonstration that breast conservation therapy (BCT) for early stage disease provides equal survival rates compared with mastectomy, a greater emphasis has been placed on cosmetic outcome without sacrificing oncologic care. Percutaneous approaches to diagnosis, such as core biopsy, have already dramatically changed the management of breast cancer. Percutaneous approaches to therapy have also been described. Percutaneous tumor ablation using various energy modalities are being investigated, including radiofrequency ablation (RFA), cryosurgery, laser, and high intensity focussed ultrasound.[1] These studies have demonstrated their potential for the complete eradication of the primary tumor without a resultant defect in the breast, minimal scar and minimal toxicity. As opposed to the coagulative necrosis (and massive protein denaturation) seen with hyperthermia, the mechanism of tumor destruction seen with cryosurgery allows for massive antigen presentation to antigen presenting cells.[2] This predisposes to the generation of a cryo-immunologic response; a systemic anti-tumor immune response which theoretically can prevent the spread and growth of micrometastasis. This may not only improve outcome, but eliminate the need for adjuvant radiation or chemotherapy in some patients. Better characterization of this cryo-immunologic response is necessary to accurately define where cryosurgery may play a role in the treatment of breast cancer. The objective of this proposed work has been to demonstrate evidence of an anti-tumor immunologic response involved in the in situ cryoablation of human breast cancer prior to surgical resection and to examine the mechanisms and components involved in the generation of a systemic anti-tumor immune response after cryosurgery in both human and murine models.

Body

The DOD physician scientist grant DAMD17-03-1-0533, "Immunologic Response to Cryoablation of Breast Cancer." began funding on July 1st, 2003. After receiving the DOD

grant, I was subsequently awarded an NIH-K08 grant. Because of conflicts between the two grants, the decision was made between myself, the University of Michigan and the DOD to relinquish the grant effective August 31, 2003.

Although the time course of the grant was brief (2 months), several aspects of the project had been initiated. The first task was to investigate the mechanism and time course involved in the development of a systemic immune response after cryoablation as compared to surgical excision in a murine model. To accomplish this, we utilized the MT-901 mammary adenocarcinoma cell line in Balb/c mice. We wanted to demonstrate that cryoablation does stimulate an immunologic effect compared to surgical resection. Mice were placed in sanitized laminar flow hoods and anesthesized. Mice were placed in a prone position and the tumor site prepared with alcohol. Procedures were performed using strict aseptic technique. For mice undergoing surgical resection, wide excision was performed with grossly negative surgical margins. After control of self-limited hemorrhage was obtained, the wound was closed with interrupted nylon sutures. For mice going undergoing cryosurgical ablation, A table-top Argon-gas-based cryoablation system (Visica Cryoablation System, Sanarus Medical, Pleasanton, California), designed to create probe temperatures of –160°C was used to perform cryoablation. Using strict aseptic technique, the overlying skin was divided and retracted away from the tumor. The freezing tip of the cryoprobe was placed into the tumor mass under direct vision and freezing was commenced through the flow of Argon gas through the probe. A thirty-second freeze was performed under direct visualization to ensure that the entire mass has been encompasses by the ice ball. A second 30-second freeze was performed if the entire mass was not frozen. The mass and probe were allowed to passively thaw and the probe was removed. The skin was then closed using interrupted Nylon sutures. All mice were placed under a warming lamp during the recovery period.

In order to demonstrate whether mice who underwent cryoablation of subcutaneous MT-

901 tumors developed an anti-tumor immune response, successfully treated mice underwent tumor rechallenge. BALB/c mice were inoculated with $3x10^6$ MT-901 cells in the right flank. On day 11, when all mice had palpable subcutaneous tumors, mice underwent either cryoablation of their tumors or surgical resection. Two weeks later, all tumor-free mice were re-inoculated on the left flank with an identical tumorigenic dose of MT-901 cells. Mice who had a recurrence of their cancer after either procedure were not included. To gauge the tumor-specific nature of this response, mice treated by cryosurgery were re-inoculated with RENCA cells. By day 10 after re-inoculation, 6 of 7 mice treated by surgical excision had grown tumors in the left flank. In mice treated by cryosurgery, only 1 of 6 mice grew tumors ($p=0.025$). Four out of 5 mice treated for MT-901 tumors by cryoablation grew RENCA tumors, suggesting this was a tumor-specific immune response.

In order to suggest a possible mechanism of the immune response, serum cytokine levels 24 hours after cryoablation and surgical excision was measured. 100μl of blood was obtained from mice one day after treatment (either cryosurgery or surgery) by a standard tail vein bleed. The specimens were centrifuged and the serum was collected and analyzed for interferon-γ (IFN-γ), interleukin-12 (IL-12), interleukin-4 (IL-4) and interleukin-10 (IL-10) using commercially available ELISAs from PharMingen. A standard curve starting at 1000 U/ML was established with 11 serial 2-fold dilutions was performed. Experimental values were computed with the use of regression analysis. A statistically significant elevation of IL-12 and IFN-γ (both Th1 cytokines was seen). No difference was seen in the Th2 cytokines IL-4 and IL-10.

At varying time points after either cryoablation or surgery, TDLN and splenocytes were removed aseptically. Lymphoid cell suspensions were prepared by mechanical dissociation. Spleen cells were treated with ammonium chloride-potassium lysis buffer (0.83% ammonium chloride, 0.1% KHCO3 and 0.004% EDTA) for 1 minute to deplete erythrocytes and were washed twice with HBSS. The cells were then activated with 1μg/mL anti-CD3 monoclonal

antibody immobilized in 24-well plates for 2 days. The cells were subsequently cultured in recombinant human IL-2 at 60 IU/ml for 3 days and then measured for IFNγ release alone or after co-culture with irradiated MT-901 cells (or RENCA cells as a control). Repeatedly, a tumor-specific response, as measured by a rise in IFNγ after co-culture with MT-901 but not RENCA, was seen in the regional lymph nodes, but this could not be demonstrated in the splenocytes. ELISPOT assay was also used to look for evidence of an anti-tumor response in the splenoctyes. Briefly, the number of IFN-γ producing cells was measured using ELISPOT assay at varying time points after either cryoablation or surgical excision. Briefly, 96-well plates were coated with antimouse IFN-γ antibody. Activated splenocytes (2x105 cells/well) were cultured for 48 hours at 37oC in a 5% CO2 incubator alone or in the presence of 5x105 irradiated MT-901 or RENCA tumor cells. After that time, wells were washed and incubated overnight at 4oC with a different clone of biotinylated anti-IFN-γ antibody. Reactions were visualized and counted using anti-biotin-AP. Again, no difference in tumor-specific cells was seen in the splenocytes at 12 days and 19 days after treatment with cryoablation or excision.

To examine NK cell function, we performed a cytotoxicity assay against Yac-1, an NK-susceptible cell line. Briefly, activated effectors were plated using round bottom 96 well plates, in 10% FBS/DMEM complete media (supplemented with HEPES, Sodium pyruvate, non-essential amino acids, 2-ME, and Pen/Strep) at (1:1) serial dilutions starting with 1 million cells/well. Yac-1 targets were labeled with 51Cr at 0.15mCi/6 million cells/530 μl for 90 minutes at 37oC under an atmosphere of 7.5% CO2. Targets were then washed three times with 40 ml media, and plated with the effectors at 0.024 million cells/100 μl/well which gives a ratio of 40:1, decreasing by twofold over the serial dilution. Wells for spontaneous and total 51Cr release (media plus targets only, and targets plus 1% Triton-X-100, respectively) were included. Plates were centrifuged at 1000 rpm for 3 minutes to increase contact of effectors and targets,

then cultured for 4 hours at 37oC under an atmosphere of 7.5% CO2. At the end of the incubation period, 20 ul of supernatant was collected and applied to a Lumaplate, dried, and counts were read using a TopCount NXT scintillation/luminescence microplate reader. The % Specific Lysis was calculated using the formula {% Specific Lysis = 100% X (Lysis- Average Spontaneous Lysis)/(Average Total Lysis - Lysis)}. The percentages of specific lysis were averaged for each data point (E:T ratio) to obtain standard deviations and graphed using Deltagraph software. The p values were determined using t-Test assuming equal variances. Mice treated by cryoablation demonstrated increased YAC-1 cytotoxicity (24.5% ± 17.3) versus both mice treated by surgery (16.5% ± 5.9, $p<.001$) or naïve Balb/c mice (9.6 % ± 1.3, $p<.001$). The difference between mice with MT-901 tumors surgically excised was significantly increased over naïve mice ($p=.002$).

We attempted to characterize the tumor infiltrating lymphocytes in cryoablated breast tumors, but to date this has been unsuccessful. Still to be performed are experiments to investigate the ability of cryoablation of a primary tumor to eradicate micrometastatic disease present secondary to IV inoculation and active immunotherapy with expanded lymphocytes from TDLN obtained from mice who underwent cryoablation, in mice with pulmonary metastases. The above describe results have suggested that cryoablation results in a brief regional response, a systemic NK cell response, but not a significant systemic T-cell or antibody response. Therefore future experiments will pursue methods to increase the immunogenicity of cryoablated breast tumors for translation to clinical use.

The second task centered on the demonstration of an immune response in patients undergoing cryosurgical ablation of early-stage breast cancer prior to surgical resection. These experiments were done as part of a multi-center trial examining the feasibility of cryoablation for early-stage breast cancer, the results of which have been published.[3] Measurements were made of cytokine levels released into the peripheral blood stream before and after cryosurgical ablation

and surgical excision alone, but were highly variable, and although some trends were seen, there no significant findings. Attempts to enumerate and characterize tumor infiltrating lymphocytes in cryo-ablated lesions after cryosurgery were unsuccessful, as there was no significant infiltrate within the time that the lesions needed to be excised as per the protocol.

The third task was to identify the development of a humoral response to cryoablation three weeks after freezing through the use of proteomics. Early results suggest that there is an increase in anti-tumor antibodies in the serum of patients treated by cryosurgery compared to lumpectomy. More data is needed and these experiments will be continued once the next protocol is initiated. We also hope to identify any evidence of a specific anti-tumor T-cell peripheral blood mononuclear cells (PBMC) and lymphocytes from the sentinel lymph node through the use of cytotoxicity and ELISPOT assays.

Key Research Accomplishments (to date)

- Early suggestion of increased serum anti-tumor antibodies in patients who underwent cryoablation of their early-stage breast cancers compared to patients treated with standard lumpectomy. More information is necessary to make any conclusions.
- Characterization of the immunologic response to cryoablation in a murine model of breast cancer. Findings included:
    - Systemic, specific anti-tumor immunity as demonstrated by resistance to rechallenge of breast cancer, but not a second cell line, after cryablation but not surgical excision.
    - A significant rise in Th1 cytokines (IL-12, IFN-gamma) but not Th2 cytokines (IL-4, IL10) after cryoablation compared to surgery.
    - A regional, specific T-cell response in the tumor draining lymph nodes after cryosurgery, but no evidence of a systemic response.

- An increase in NK cell activity after cryoablation compared to surgical excision.

Reportable Outcomes

Sabel MS, Nehs M, Su G, Lowler K, Ferrara JLM, Chang AE. Immunologic response to cryoablation of breast cancer. Manuscript in preparation.

Conclusions

Percutaneous tumor ablation using various energy modalities, including radiofrequency ablation (RFA), cryosurgery, laser, and high intensity focussed ultrasound, show significant promise towards treating small breast cancers without a resultant defect in the breast, minimal scar and minimal toxicity. However, cryosurgery holds more potential in that in addition to ablating the tumor, there may be the generation of a cryo-immunologic response; a systemic anti-tumor immune response which theoretically can prevent the spread and growth of micrometastasis. In the murine experiments described above, there is clearly the generation of a tumor-specific response, that appears to be primarily induced by Th1 cytokines resulting in T-cell and NK cell activation. However, this reaction is limited. These experiments suggest that the cryoimmunologic response will need to be augmented by adjuvant immunotherapies if they are to be clinically relevant. The potential for this is great, as this may not only reduce recurrence rates and improve outcome, but eliminate the need for adjuvant therapies in some patients.

Further murine experiments are necessary to study the best ways to augment this immune response. It is also imperative to continue ongoing human trials to examine any potential immunologic benefit of cryoablation of small breast tumors.

References.

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3. Sabel MS, Kaufman CS, Whitworth P, et al. Cryoablation of Early-Stage Breast Cancer: Work-in-Progress Report of a Multi-Institutional Trial. Ann Surg Oncol 2004; 11(5):542-9.